Investigating The Proteome-guided Diagnostic And Therapeutic Approach For *Japanese Encephalitis*

SHORT SYNOPSIS

Submitted in partial fulfilment of the requirement of the degree of

DOCTOR OF PHILOSOPHY

to

Manav Rachna International Institute of Research and Studies (Deemed to be University)

by

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Certificate

I hereby declare that the short synopsis " Investigating The Proteome-guided Diagnostic And Therapeutic Approach For *Japanese Encephalitis* " being submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Biotechnology under the Department of Biotechnology of the Manav Rachna International Institute of Research and Studies, Faridabad, during the academic year 2024. It is a bonafide record of original work carried out under the supervision of Dr. Rashmi Rameshwari. It has not been submitted in full or in part to any other university or institute for a degree or diploma. Further, it is certified that the short synopsis is plagiarism-free, and the proof has been submitted.

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ABSTRACT

Japanese encephalitis (JE), caused by the *Japanese encephalitis* virus (JEV), is a common mosquito-borne infection in the region of Asia, known for its severe neurological repercussions. The diagnostic picture is confounded by symptoms that overlap with other frequent illnesses in Asia-Pacific. Using label-free quantitative mass spectrometry, unique JE protein signatures in human cerebrospinal fluid were identified, expediting rapid diagnostic test (RDT) development and deepening our understanding of the host immune response for treatment insights. Employing semiquantitative proteomics, drug repurposing constructs a dynamic protein signature associated with JEV infection in mouse brains. Connectivity Map (CMap) analysis then refines drug candidates, selecting Tipifarnib, Ly303511, and MDL11939 to assess in JEV-infected mouse neuroblastoma cells and C57BL/6 mice. Additionally, cell-free therapy employs human bone marrow-derived extracellular vesicles (BM-EVs) to explore their impact on JEV replication and pathogenesis in primary neuronal stem cells and murine models. Expected outcomes include mitigating JEV-induced inflammation and fatality in mice via BM-EV treatment. In contrast, drug validation against molecular expression changes in JEV-infected mouse brains offers alternative therapeutic avenues.

Keywords - *Japanese Encephalitis* Virus, Mass Spectrometry, Mesenchymal Stem Cells, Extracellular Vesicles, Drug -Repurposing.

List of Abbreviations:

JEV - *Japanese encephalitis* Virus

BBB – Blood Brain Barrier

hMSCs – Human Mesenchymal Stem Cells

FDA – Food and Drugs Administration

CNS – Central Nervous System

CSF – Cerebral Spinal Fluid

EVs – Extracellular Vesicles

CD – Cluster of Differentiation

ELISA – Enzyme-Linked Immunosorbent Assay

C17.2 Cells - Mouse Neuronal Progenitor Cells

NVBDCP - National Vector Borne Disease Control Programme

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1. Description Of Broad Area

Acute encephalitis syndrome (AES) outbreaks in India are a significant public health concern, impacting thousands of individuals annually. *Japanese encephalitis* virus (JEV) is believed to be the primary cause of acute encephalitis syndrome. Worldwide, an estimated 68,000 clinical cases cause around 13600-24000 fatalities each year. In response to a 2005 outbreak that resulted in 5737 cases and 1344 deaths, the Government of India implemented JEV vaccination in affected areas. However, Between 2014 and 2017, the NVBDCP reported 10867-13 672 cases of acute encephalitis syndrome in India, suggesting no decrease in the overall prevalence. Despite being practiced vaccination, JEV cases persist and cause mortality. Hence, with time, the advancement, and the pathogenicity of the virus, vaccines show varied safety efficacy. The situation demands an alternative to vaccines to treat and cure the suffering. Many approaches have been utilized to control the pathogenicity to date. However, no effective alternative was found. Hence, MSC-derived exosome-based therapy has shown protective effects in varied disease pathogenesis, but, to date, it has not been tested in the case of JEV. Evidence supports its candidature as the safest and most effective alternative to combat the JEV disease pathogenesis. This study will utilize the C57BL/6 mice as a JEV model to check the effectiveness of MSC-derived exosomes in controlling viral replication and pathogenesis.

Introduction

JEV, a mosquito-borne flavivirus, is a prominent cause of neurological illness throughout Asia and the Pacific, often known as Japanese encephalitis (JE). The first known instance of Japanese encephalitis viral disease (JE) in Japan occurred in 1871 (Sharma et al., 2021). Globally, JE affects around 68,000 clinical cases each year, killing 13,600 to 20,400 people [1]. It mainly infects children below the age of <14. The infant displayed a range of symptoms, beginning with headache, vomiting, and fever and progressing to severe encephalitis due to a lack of care. A lack of treatment or a delay in diagnosis kills 20-30% of patients. Approximately 40 - 50% of patients acquire lifetime severe neurological sequelae [2].

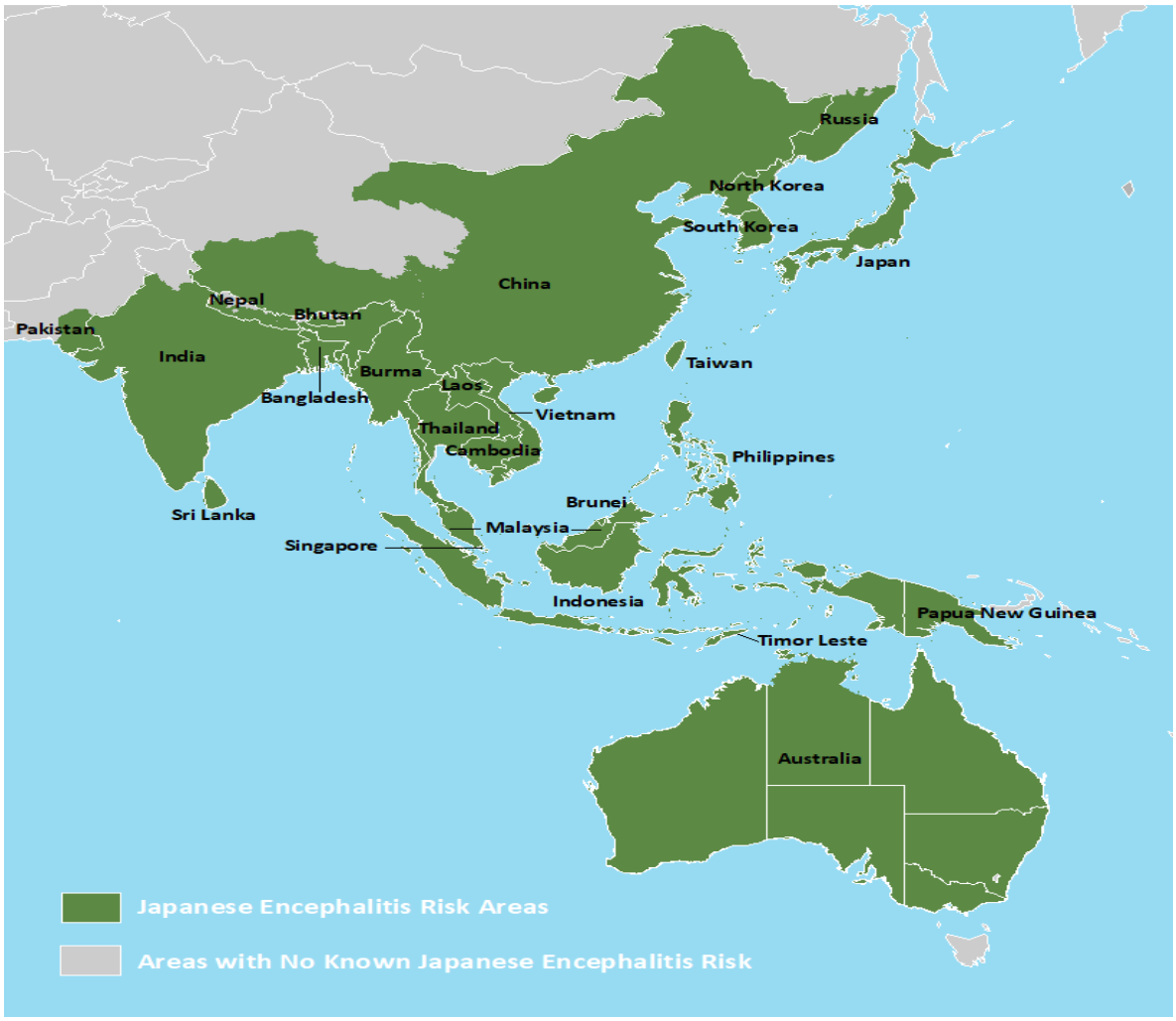

Figure 1: The Centers for Disease Control and Prevention (CDC) has issued a global map showing places at risk of Japanese encephalitis.

Figure 2: Map showing the distribution of JEV in India. adapted from [3].

1.1 JEV Structure

JEV is a flavivirus with an envelope of roughly 50 nm in diameter. The genome is approximately 11 kb long and has a positive single-stranded RNA genome. The 5' end is capped, but the 3' is not polyadenylated. The genome has one open reading frame (ORF). Flanked by 5’ and 3’ non-translated regions (NTRs). The Capsid (C) protein is a fundamental

protein that forms the viral nucleocapsid by interacting with RNA [4], [5]. The glycoprotein Envelope (E) is crucial. It serves a role in viral attachment and fusion. JEV's near-atomic structure reveals various structural variables connected with viral stability and neurovirulence. [6]. Protease hydrolysis creates the M protein from prM (precursor form), which results in viral particle maturation [7]. Non-structural proteins are vital for viral propagation and function alongside host proteins [8].

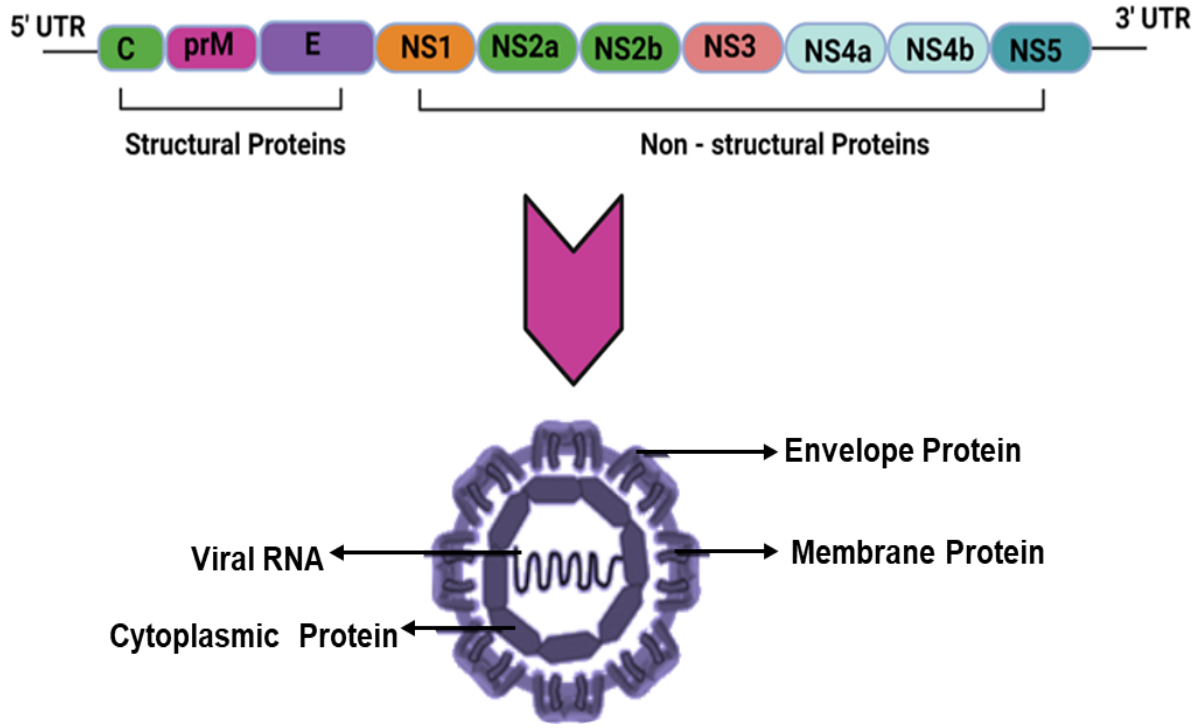

Figure 3: *Japanese encephalitis* Virus genomic representation and structure arrangement.

1.2 JEV Transmission

JEV is spread to humans via bites from infected mosquitoes (Culex tritaeniorrhynchus and Culex vishnui). It is a virus primarily found in rural and peri-urban settings [9]. It is transmitted

mainly during the warm season in temperate Asia and intensifies during the rainy and pre-harvest periods in rice-cultivating regions. Once the mosquito bites, the virus first infects resident dermal cells and local lymph nodes. This leads to primary asymptomatic viremia. Secondly, The virus spreads to many bodily organs via the efferent lymphatic system and the hematogenous pathway [9], [10].

1.3 Mechanism of JEV neuroinvasion

In the peripheral system, JEV replicates in Ly6Chi monocytes, expressing CCR2, and migrates to the central nervous system, contributing to inflammation [11]. Evidence suggests that viruses enter the CNS via the BBB, impacting brain microvasculature endothelial cells and neuro-vasculature units while maintaining homeostasis via immune cell and soluble chemical transport [12]. JEV enters the central nervous system (CNS) by four mechanisms: virus proliferation, passive transport, diapedesis, peripheral nervous system transport, and rupture of the BBB via virus-induced inflammatory mediators. The most common method is virus-induced apical and basolateral BBB cell inflammation. After reaching the CNS, the virus replicates in neurons and glial cells and releases chemokines and cytokines, further contributing to the neuroinflammation paradigm [10], [13]. Mesenchymal stem cells (MSCs), known for their varied renewal powers, have demonstrated extraordinary efficiency in battling the Japanese encephalitis virus (JEV) in a complex encephalitis model [14]. However, they had some limitations that needed to be overcome. No licensed medications for treating JEV infection exist, and vaccines are ineffective against specific JEV strains. Drug repurposing, an innovative method investigating alternate applications for existing medications, has recently gained popularity in creating new antiviral treatments. A critical feature of this technique is the difficult process of identifying host proteins that are important in the course of JEV infection. Utilizing these proteins as possible targets is the foundation of repurposing JEV therapy [15].

1.4 Drug Re-purposing

Drug repurposing is uncovering new uses for licensed or experimental pharmaceuticals that go beyond their original medical indications, providing a systematic approach to discovering alternative applications for these therapies [16]. Drug repurposing has substantial advantages

over generating new drugs for specific indications. First, repurposed medications have a documented safety profile based on preclinical research and human trials, reducing the probability of failure in following effectiveness trials. Second, comprehensive preclinical research, safety review, and formulation development have already been completed, which speeds up the medication development process and results in shorter timescales. Third, the financial investment necessary for repurposing is often cheaper, depending on the stage of development and the procedure. These advantages reduce risks and provide a quicker return on spending on the development of reused medications [17]–[19].

1.5 Mesenchymal Stem cells

Mesenchymal stem cells (MSCs) are the multipotential/progenitor population belonging to the class of adult stem cells. MSCs are well known for their potential to regenerate, self-renewal, and multilineage differentiation. They were found in bone marrow but later in other tissues– umbilical cord, adipose tissue, placenta, and teeth. Characteristics were different based on their extraction source but must possess three main characteristics, as the International Society for Cellular Therapy (ISCT) [20].

(I). Adherence in nature when grown in an *in-vitro* system.

(II). The sample stained positively for surface antigen markers CD73, CD90, and CD105 but negatively for CD45, CD34, CD14 or CD11B, CD19, and HLA-DR.

(III). These cells have a remarkable capacity to develop into mesodermal lineages. Furthermore, they can differentiate into ectodermal and endodermal lineages, including brain cells, cardiomyocytes, hepatocytes, and endothelial cells [21].

Over the last few decades, stem cell-based therapy has gained the limelight in the field of regenerative medicines and also shows promising effects in cancer, neurodegenerative disease, aging, cardiac ischemia, cartilage, bone diseases, and various infectious diseases [21]. MSCs exhibit the capacity to move to the damaged site, allowing for immediate intervention. Furthermore, MSCs use paracrine pathways to coordinate their influence, such as releasing growth factors, cytokines, and Microvesicles, particularly via exosomes [22]. Exosomes,

widely documented in many disorders, have also been shown to have protective effects on mesenchymal stem cells.

1.6 Extracellular Vesicles (EVs)

Exosomes are a class of extracellular vesicles with sizes ranging from 20 to 150 nm. They are released by eukaryotic cells and were formerly considered waste products. Recent research, however, has revealed that Exosomes operate as a unique mode of intercellular communication and influence various biological processes. Their functioning depends on delivering bioactive payloads to target cells, including lipids, metabolites, proteins, and nucleic acids [23], [24].

2. Literature Review

Japanese encephalitis, a severe neuroinvasive disease, is caused by the flavivirus *Japanese encephalitis* virus (JEV), transmitted by Culex mosquito vectors. It is prevalent across Asia-Pacific, and its potential for worldwide spread is significant. The symptoms range from mild febrile bouts to severe encephalitis and death, demonstrating the disease's significant neuroinvasiveness. Disease prognosis is closely linked to several signaling cascades, including cellular stress responses and host immune-inflammatory reactions. Strategic regulation of these pathways is a possible treatment strategy for alleviating the disease's long-term problems [10]. Despite vaccine research and development, no antivirals are available to treat JEV-related disorders. Mesenchymal stem cells (MSCs) have demonstrated therapeutic potential for central nervous system illnesses. In vivo, investigations revealed that MSC therapy reduced JEV-induced mortality and enhanced JE recovery. In Neuro2a cells, MSCs altered microglia switching, increased neuron survival, and lowered viral load [25]. Japanese encephalitis is a severe neuroinvasive condition caused by infection with the flavivirus Japanese encephalitis virus (JEV), which is spread by Culex mosquito vectors. Its prevalence is widespread throughout Asia-Pacific, and it has enormous worldwide potential. The symptoms range from mild febrile bouts to severe encephalitis and death, emphasizing the disease's significant neuroinvasive proclivity. Disease prognosis is tightly linked to many signaling cascades, such as cellular stress and host immune-inflammatory responses. Strategic manipulation of these pathways emerges as a possible treatment approach for tackling chronic disease problems [26]. In contrast, the influence of MSCs-EVs on neuroinflammation has been studied. Initially, a mouse model showed that giving MSCs might reduce neuroinflammatory reactions induced by lipopolysaccharide (LPS). MSCs were later discovered to alter M1 and M2 polarization both

in vitro and in vivo. Furthermore, the critical function of TSG-6 in mediating MSC effects on microglial polarization was found. This study elucidates that mesenchymal stem cells (MSCs) facilitate M2 polarization of microglia through TSG-6, thereby eliciting anti-neuroinflammatory effects [27], [28] The intravenous infusion of MSCs to BALB/c mice infected with the highly pathogenic avian influenza A/H5N1 virus produced noteworthy results. Treatment dramatically reduced lung damage, decreased lung alveolar protein levels, and improved the PaO2/FiO2 ratio and histological markers. MSC-treated mice showed reduced levels of NF-κB, RAGE, TNFα, and IL-1β but increased expressions of Sftpc and Aqp5+. MSC treatment efficiently avoided future lung damage and inflammation but did not directly influence viral proliferation or mice morbidity and death rates.

Drug repurposing is an advanced way to utilize the available drugs for novel causes other than the classical ones. Five mosquito-borne viral illnesses were studied using a computational drug repositioning method: dengue, zika, WNV, JEV, and Chikungunya. Signature molecules and pathways were discovered, as well as 77 therapeutic candidates and 146 proteins. The study looked at the interaction between medications, target proteins, and the five viruses, categorizing drug possibilities based on target protein [29]. Another study reports that High throughput screening was performed to find five suitable drugs for JEV. Drugs capable of inhibiting JEV under >10 selective index. All five drugs are calcium inhibitors. Further testing was done *in vivo* to check the effect of shortlisted drugs. Lastly, they found that pathogenesis is controlled in mice and concluded that cytoplasmic calcium plays a pivotal role in JEV pathogenesis; hence cytoplasmic calcium could be a possible target for antiviral treatment for JEV [30], [31]. Another study looks at the binding locations of four flaviviral RdRp enzymes, which can provide insights into molecular interactions. These findings aid in discovering effective molecules capable of blocking the viral protein as a universal inhibitor. The study also proposes a mechanism of inhibition for identified small molecules towards the RdRp of flavivirus. A transcriptomic-based technique was used to evaluate sixteen medicines for preventive, virucidal, and therapeutic activities against the Dengue virus (DENV). Notably, five medicines, including resveratrol, doxorubicin, lomibuvir, elvitegravir, and enalaprilat, shown strong anti-DENV action. These drugs interacted with DENV protein targets, resulting in decreased DENV-2 production and showing promise as possible therapies [32]. The Zika Virus (ZIKV) is a significant danger to worldwide public health, necessitating the development of therapies. A study targeted Non-Structural Protein 5 (NS5) and tested 2895 FDA-approved chemicals. Ceforanide, Squanavir, Amcinonide, Cefpiramide, and Olmesartan_Medoxomil were chosen

for Molecular Dynamic Simulations (MDS) research. According to the binding energy estimations, the most stable molecules for binding to NS5 are Cefpiramide and Olmesartan_Medoxomil. These drugs have been examined on pharmacokinetic and pharmacodynamic parameters, and clinical trials for their potential application in treating ZIKV patients may be recommended [33]. Five mosquito-borne viral illnesses were studied using a computational drug repositioning method: dengue, zika, WNV, JEV, and Chikungunya. Signature molecules and pathways were discovered, as well as 77 therapeutic candidates and 146 proteins. The study looked at the interaction between medications, target proteins, and the five viruses, categorizing drug possibilities based on target protein [29].

3. Research Gap and Problem Statement

- *Japanese encephalitis* (JE) remains a significant worldwide health concern, with no accurate diagnosis and treatment procedures. Given the high morbidity and death rates among symptomatic persons, there is an urgent need for thorough academic research into potential treatment options.
- *Japanese encephalitis* symptoms possess similarities to other non-JE infections and hence become difficult to diagnose timely. the differential diagnosis molecule or protein is needed for precision diagnosis of the disease
- To date, no antiviral is available for disease management; hence, without delay, there is an urgent need to discover a new drug; in this context, drug repurposing is a better approach to limit the consumption of time and money—utilization of an FDA-approved drug capable of inducing antiviral and neurogenesis-promoting activity. Pilot studies of approved medications with animal efficacy are required to investigate effective therapeutics.
- Mesenchymal stem cells (MSCs) effectively treat CNS disorders and JEV by reducing inflammation and other processes. However, they have limitations, such as being imprisoned in peripheral organs, failing to cross the BBB to mediate immediate action, and many more. To overcome these limitations, mesenchymal stem cell-derived extracellular vesicles (exosomes) are suggested.

Following immunization campaigns, *Japanese encephalitis* virus (JEV) remains a significant public health issue, particularly in endemic Asian locations, due to differences

in vaccination effectiveness and diagnostic problems. The diagnostic picture is complicated by symptom overlap with different viruses, demanding an exact diagnosis capable of distinguishing JEV from related disorders. Furthermore, the complexities of pharmacological research provide substantial challenges in developing effective JEV therapies.

4. Objectives of the proposed study

1: To investigate the changes in proteomic profiles of cerebrospinal fluid from *Japanese encephalitis* virus patients.

2: To apply a proteome-guided approach to enhance antiviral efficacy and strengthen immune responses against *Japanese encephalitis* by identifying key viral and host proteins.

3: To understand the effect of mesenchymal stem cell-derived exosomes for counteract *Japanese encephalitis* virus Pathogenesis.

5. Proposed Methodology to Address Objective.

Objective 1: To investigate the changes in proteomic profiles of cerebrospinal fluid from *Japanese encephalitis* virus patients.

CSF from the JEV-positive patients collected during 2019 -2020 (With patient consent) will be utilized to study the protein expression change compared to non-AES patient CSF. The LC-MS technique will identify the protein and run it on Spectronout for further analysis. The findings will be further validated using ELISA.

Objective 2: To apply a proteome-guided approach to enhance antiviral efficacy and strengthen immune responses against *Japanese encephalitis* by identifying key viral and host proteins.

C57BL/6 mice will be infected with JEV, and after symptom onset, the brain will be Harvested. The harvested brain sample will be further processed for Mass Spectrometry and data analysis to identify relevant drugs. Identified drugs will be tested further in vitro and In vivo model systems for their toxicity profile. After quantifying the drug's toxicity, further study will be

performed on the ex vivo and in vivo models of JEV infection. The following parameters will be checked: Mice treated with JEV were monitored daily for body weight and clinical scoring. The dye will be used to test BBB permeability, and viral load will be determined by qRT-PCR and an NS3 protein Western blot. QRT-PCR will quantify inflammatory gene expression, and pathogenesis will be assessed using H&E staining. Additionally, cytokine and immune cell profiles will be evaluated using FACS.

Objective 3: To understand the effect of mesenchymal stem cell-derived exosomes for counteract *Japanese encephalitis* virus Pathogenesis.

Isolation and culture of hMSCs from Bone Marrow (BM) from a healthy individual and their EVs -The MSCs will be cultured in serum-free media for 48h. Post that, conditioned media collected from the MSCs culture will be further exercised for EV isolation using ultracentrifugation. BM-EVs will be further utilized to investigate their neurogenic potential and capacity to limit viral pathogenesis in both the model system *in vitro* and *in vivo* using C17.2 cell lines and C57BL/6 mice. The following parameters will be checked - EV uptake - PKH67 dyes-stained BM-EVs will be cultured with C17.2 cells and neurosphere for different time points like 12, 24, and 36, post the uptake will be monitored by using confocal microscopy and flow cytometry. Neurogenesis by qRT-PCR, Viral load in *in vitro* system by qRT-PCR, and Western blotting, C57BL/6 mice will be infected with Jev at $1X10^7$ plaque-forming unit by intraperitoneal (IP) administration. Afterward, Mice will be given 50µg of protein equivalent EVs twice daily (12 hours post-infection) for 100µg of BM-EVs every 24 hours. Weight and survival longevity will be monitored, RT-PCR and Western blotting will monitor Evans dye, viral load, cytokine profile will be checked, and immune cell infiltration by FACS will check BBB permeability.

6. Expected Outcome of the Research

This study would provide valuable information regarding the efficacy of MSC-derived extracellular vesicles and drug repurposing approach in regulating viral load and immunomodulatory factors in case of viral-induced neurodegenerative disease. The integrative molecular and proteomics approach will help better understand the biological relevance of proteins modulated during infection and lead to better strategies to overcome the disease outcome.

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Summary

Investigating The Proteome-guided Diagnostic And Therapeutic Approach For *Japanese Encephalitis*

SHORT SYNOPSIS

by

Naina Soni

Registration number: 22/Ph.D/S/039

Under the supervision of

Dr. Rashmi Rameshwari
Associate Professor
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ABSTRACT

Japanese encephalitis (JE), caused by the *Japanese encephalitis* virus (JEV), is a common mosquito-borne infection in the region of Asia, known for its severe neurological repercussions. The diagnostic picture is confounded by symptoms that overlap with other frequent illnesses in Asia-Pacific. Using label-free quantitative mass spectrometry, unique JE protein signatures in human cerebrospinal fluid were identified, expediting rapid diagnostic test (RDT) development and deepening our understanding of the host immune response for treatment insights. Employing semiquantitative proteomics, drug repurposing constructs a dynamic protein signature associated with JEV infection in mouse brains.

Connectivity Map (CMap) analysis then refines drug candidates, selecting Tipifarnib, Ly303511, and MDL11939 to assess in JEV-infected mouse neuroblastoma cells and C57BL/6 mice. Additionally, cell-free therapy employs human bone marrow-derived extracellular vesicles (BM-EVs) to explore their impact on JEV replication and pathogenesis in primary neuronal stem cells and murine models. Expected outcomes include mitigating JEV-induced inflammation and fatality in mice via BM-EV treatment. In contrast, drug validation against molecular expression changes in JEV-infected mouse brains offers alternative therapeutic avenues.

Keywords - *Japanese Encephalitis* Virus, Mass Spectrometry, Mesenchymal Stem Cells, Extracellular Vesicles, Drug -Repurposing.

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